EMBO reports

The heptad repeat domain 1 of Mitofusin has membrane destabilization function in mitochondrial fusion

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 21 July 2017

Thank you for the submission of your manuscript to our journal. I am sorry for the delay in getting back to you; it is not easy to find referees during the summer season, and we also give them more time for peer-review at this time of the year. I have taken over the handling of your manuscript from my colleague Martina, since she is not in the office.

We have now received the complete set of referee reports that is pasted below.
As you will see, the referees acknowledge that the findings are potentially interesting and important. However, they also have a number of suggestions for how the data should be strengthened and the study further improved. In their cross-comments referees 1 and 3 agree with each others' concerns, and all concerns therefore need to be successfully addressed for publication of the study here.

We would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further. Given the 8 main figures, I suggest that you layout your manuscript as a full article. Please note that all materials and methods need to be included in the main manuscript file. Supplementary figures and tables are called Expanded View

(EV) at EMBO press now, but we can only offer a maximum of 5 EV figures per manuscript, in addition to EV tables. Additional data would need to be included in the Appendix file. Alternatively, all supplementary data could be in the Appendix file. Please see our guide to authors online for more information.

Regarding data quantification, please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information must be provided in the figure legends. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

This study provides new and unexpected insight into the molecular contribution of the heptad repeat domains of the mammalian mitofusin proteins in driving bilayer fusion. While we have known for nearly 20 years that these proteins are highly conserved and essential for mitochondrial fusion, very little insight has been gained concerning the precise mechanisms of fusion. Recent crystal structures of a "mini-Mfn1" protein indicated that a 4 helix bundle containing HR1 and HR2 are adjacent to the GTPase domain, which can bind in trans. However, this structure was generated upon deletion of nearly 300 residues, and linked HR1 to HR2 in a rather artificial manner. Therefore, no real insight could be gleaned from these structures concerning the potentially dynamic roles of these two domains with lipid bilayers. Work presented here provides compelling evidence that these two domains have very distinct roles in membrane docking and fusion, providing a basis for future structure/function work to understand how this model may hold in the context of full length Mfns. Overall, I think it is important work that is well controlled and highly informative. There are, however some questions and concerns that should be addressed, particularly in adding more kinetic and titration data that can help dissect some of the processes more clearly.
• The authors examined the importance of HR1 and HR2 by expressing truncated forms of MFN1 in rescuing Mfn1 KO fibroblasts. It has previously been shown that the truncation of the C-terminal domain of MFN2 resulted in the mislocalization of Mfn2 (Rojo et al, 2002, JCS). It is important to confirm import and insertion of these truncation constructs, and to directly compare protein expression levels in the transfection experiment.

• The power of the reconstitution experiments is clear, however there is an important limitation in that a major lipid species could not be included (PE) due to the interference with maleimide conjugation of the recombinant peptides. Could the authors express a minimal TMD anchored HR1-FLAG (or equivalent) in mammalian cells for immunoisolation and reconstitution into more physiological liposomes that could then be used in a dequenching assay (either homotypic or heterotypic with naked liposomes)? I realize this may be a complex experiment, particularly if the expressed proteins may find strong binding partners within the cell. But it could work well if the isolated proteins are carefully washed in high salt, etc.

• There is an essential control that should be done throughout the study - which is to mix HR1 with HR2 containing liposomes. There are many who would argue that these should also be able to bind in trans (notably the recent study by the Dorn lab PMID:27775718), so this pairing should be included.

• It is not clear what the protein/lipid ratio is for the cysteine-HR1/2 added to the liposomes for fusion or tethering. It is important to include a titration of HR1/2 to determine the stoichiometry that may drive fusion and whether this makes sense with endogenous expression within mitochondria. Previous experiments with Atlasin-reconstituted liposomes included these kinds of experiments, which were very informative (Orso et al. 2009; Lee et al. 2015)

• The authors should really attempt to examine the size of any complexes that may form between the C18 and C45 conjugated peptide. Do they stay as monomers or assemble into larger structures? These could be examined by BN-PAGE or sizing columns. Early work has established distinct complexes in cis vs trans (PMID:15572413), and more recent work by the Cohen lab has shown the large ring structures (that the authors cite in the discussion). It should be straightforward to test the types of assemblies formed by the HR1 vs HR2 conjugates in the bilayer.

• The fact that HR1 mediates lipid mixing with both C18 and C45 anchored HR1 is intriguing, and to this reviewer suggests that hemifusion is the primary mode of action. In eliminating fluorescence of outer leaflet, the authors revealed 40% of liposomes underwent full fusion while 60% only hemifusion. Again, I think that kinetic analysis of fusion in this quenched outer leaflet mode may be helpful if, for example, HR1 anchors the lipids to initate hemifusion, which may spontaneously resolve into full fusion after a certain time. This is distinct from the SNAREs (as the authors explain), which only fuse C45 anchored liposomes.

• Related to that last comment, if kinetic and/or titrations were performed it could be possible to capture the hemifused states by EM. Currently this is inferred from the fluorescence and I think it could provide an important mechanistic advance.

• The authors tested whether HR1 and HR2 can tether liposomes upon incubating biotinylated, non-fluorescent liposomes with fluorescent liposomes, in the presence of streptavidin beads, followed by fluorescence that is pulled down with the beads (Fig4A). The results are not very convincing, with large error bars indicating low significance, although the incubation was done overnight. The inclusion of SNARE pairs as a positive control is very important, but again, a kinetic and titration analysis would really help to understand the physiological significance of this tethering. Very early work by Mihara's group suggested that Mfn1 but not Mfn2 could tether mitochondria, and that the hydrolysis and exchange rates of Mfn1 were much higher than Mfn2 (PMID:15572413). The GTP dependence of the tethering shown back then is consistent with the model emerging from the crystal structure that the tethering is done through the head to head binding of the GTPase domains in trans. From all of this, I'm not convinced that the HR domains would really be efficient tethers. Therefore, it is important to expand this figure with additional kinetic analysis.

• The authors examined the tethering mediated by HR1/2 by EM. However, it isn't clear how "clustering" is defined in terms of nm spacing between membranes. The field has a concept of 20-30nm as a distance between opposing membranes that are functionally tethered. Are these tethers dilution resistant? Perhaps I missed that point.

Referee #2:

Authors examined the biochemical/biophysical properties of the HR1 domain of mitofusin, which is important for mitochondrial outer membrane fusion and for maintenance of mitochondrial function. They show that (i) HR1 domain is required for mitofusin-mediated mitochondrial fusion in cultured cells, (ii) HR1 domain induces membrane fusion and docking using in vitro fusion assay, although HR2 only mediates docking, (iii) membrane-anchored HR1 induced heterotypic fusion with protein-free liposome, (iv) membrane binding and perturbation properties of HR1 are from its amphipathic helix structure. The authors propose a model in which HR1 interacts with the lipid membrane and then, brings membrane in close and perturbs the lipid bilayer structure to drive membrane fusion.

The subject of this study is highly relevant and interesting because little is known about the mechanism of membrane fusion by mitofusin proteins. Observing membrane fusion and docking with HR1 peptide is partly significant, however, this study add less to molecular understanding of mitochondrial fusion, since this study is very limited in HR1 domain, and these data are just descriptive

Critical issues in this manuscript are: (1) The membrane-destabilizing property of HR1 is speculative - no direct experimental evidence is provided. (2) Significance of the HR1 mediated liposome docking by homotypic and heterotypic in Mfn1-mediated mitochondrial fusion are not clear.
Other points: (1) It is not clear how much protein was used for experiments in Fig.2-7. I did not find such important information in main text. (2) It is not clear why both synthetic and recombinant HR1 are used in this study.

Referee #3:

The authors present a very nice dissection of the promotion of membrane fusion by MFN1 HR1.

Specific comments:

1) Fig. 4B - it would be good to have a picture here of the C45 image, which is the negative control, in addition to the two experimental conditions, +HR1 and +HR2. It's not possible to interpret the significance of the docked and enlarged vesicles without having the starting conditions / negative control to compare them to.

2) Fig. 7B - negative results, in particular, require validation of the experimental details, to attempt to rule out as many trivial explanations as possible. In this instance, the authors show that excision of the 18-amino acid amphipathic helix eliminates the fusion promoting capacity of Mfn1. However, there is no validation of this construct, e.g. is it expressed at similar levels (western blotting) and does it subcellularly localize correctly (microscopy)? Although the findings are consistent with the proposed model, they could be strengthened as described above and using other approaches (e.g. the assays used in prior figures).

3) More generally, while the work is carefully performed using PC liposomes as described, PC liposomes are far from physiological. This is evidenced by the rescue of fusion using PE with larger liposomes for HR1. One wonders how HR2 might behave in this assay, or the mutant lacking the amphipathic helix. Some of the other lipids reported to be present in high quantities in contact sites, such as CL (up to 25% of the membrane lipids there) or phosphatidic acid (another cone shaped lipid) are thought to have profound effects on membrane structure and order. Whether the well-described findings here would remain equally true with more physiological membrane targets seems unknowable without testing (e.g., whether HR2 would exhibit fusogenic activity).

4) Is there a cognate amphipathic helix in HR2?

1st Revision - authors' response 9 December 2017

Point-by-point answer to all referees' comments

Referee #1:

This study provides new and unexpected insight into the molecular contribution of the heptad repeat domains of the mammalian Mitofusin proteins in driving bilayer fusion. While we have known for nearly 20 years that these proteins are highly conserved and essential for mitochondrial fusion, very little insight has been gained concerning the precise mechanisms of fusion. Recent crystal structures of a "mini-Mfn1" protein indicated that a 4 helix bundle containing HR1 and HR2 are adjacent to the GTPase domain, which can bind in trans. However, this structure was generated upon deletion of nearly 300 residues, and linked HR1 to HR2 in a rather artificial manner. Therefore, no real insight could be gleaned from these structures concerning the potentially dynamic roles of these two domains with lipid bilayers. Work presented here provides compelling evidence that these two domains have very distinct roles in membrane docking and fusion, providing a basis for future structure/function work to understand how this model may hold in the context of full length Mfns. Overall, I think it is important work that is well controlled and highly informative. There are, however some questions and concerns that should be addressed, particularly in adding more kinetic and titration data that can help dissect some of the processes more clearly.

Q1: The authors examined the importance of HR1 and HR2 by expressing truncated forms of MFN1 in rescuing Mfn1 KO fibroblasts. It has previously been shown that the truncation of the C-terminal domain of MFN2 resulted in the mislocalization of Mfn2 (Rojo et al, 2002, JCS). It is important to confirm import and insertion of these truncation constructs, and to directly compare protein expression levels in the transfection experiment.

A1: This is an important point. To answer this comment, we have performed additional transfection experiments of the various Myc-tagged Mfn1 variants used in this study and characterized, for the same cell populations, both the protein expression level (by Western Blot using an anti-Myc antibody) and the protein localization at the mitochondrial surface (by immunofluorescence microscopy probing for its co-localization with an EGFP molecule targeted to the mitochondrial matrix). Western blot experiments revealed that all Mfn1 variants were expressed at similar levels when compared with tubulin, although the expression levels appeared slightly higher for wild-type Mfn1 than for heptad repeat or amphipathic helix deleted mutants (Fig. R1, top left). Co-localization experiments (quantified by the Pearson's correlation coefficient, PCC) showed that the PCC of images of Mfn1 variants and mitochondrial EGFP had values close to or larger than 0.7, proving that all Mfn1 variants were efficiently targeted to mitochondria (Fig. R1, top right). Interestingly, both the HR1 deleted mutant and the amphipathic helix deleted mutant displayed slightly lower PCC values (0.74 and 0.70, respectively) compared to the wild-type Mfn1 and the HR2 deleted mutant (0.83 for both variants). It is thus possible that the amphipathic helix of HR1, in addition to its role in mitochondrial fusion, has also a function in mitochondrial targeting as recently shown for an amphipathic helix located between the transmembrane domain and the HR2 domain of Mfn1 (Huang et al, PNAS, 2017). To investigate whether Mfn1 variants were correctly inserted into the outer mitochondrial membrane, we have also performed pilot protein extraction experiments on mitochondria purified from Mfn1 KO MEFs transfected with the various Mfn1 variants. Purified mitochondria were resuspended in 10 mM HEPES, 75 mM Sucrose, 225 mM Manitol and treated with 100 mM NaCl alone (control) or in combination with100 mM Na_2C0_3 (known to disrupt ionic intermolecular interactions and thus remove peripheral membrane proteins) or 1% Triton X-100 (to solubilize membranes and extract all embedded proteins). After incubation, samples were pelleted and the pellets were analyzed by Western Blot (Fig. R1, bottom). This preliminary set of experiments showed that all Mfn1 variants were resistant to treatment with sodium bicarbonate, indicating that they were all inserted into the outer mitochondrial membrane *via* their transmembrane domain.

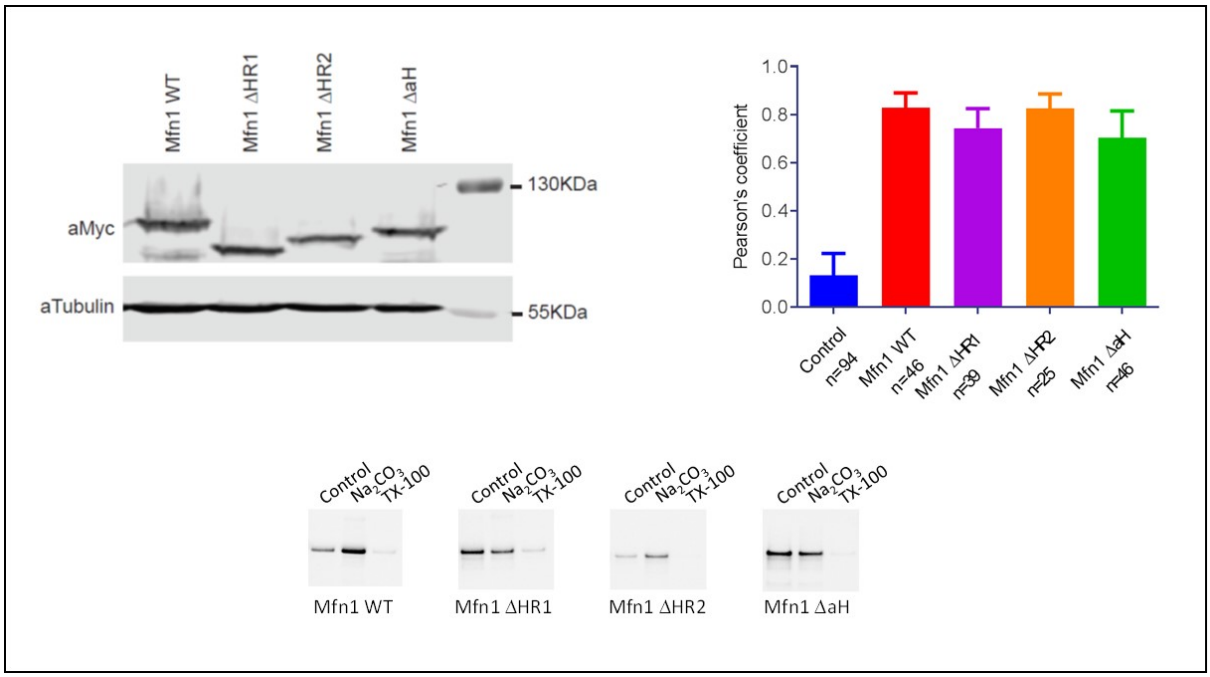

Figure R1.
Data of this figure (top panels) is shown in Figure S2 and described on pages 6-7 of the new manuscript

Q2: The power of the reconstitution experiments is clear, however there is an important limitation in that a major lipid species could not be included (PE) due to the interference with maleimide conjugation of the recombinant peptides. Could the authors express a minimal TMD anchored HR1-FLAG (or equivalent) in mammalian cells for immunoisolation and reconstitution into more physiological liposomes that could then be used in a dequenching assay (either homotypic or heterotypic with naked liposomes)? I realize this may be a complex experiment, particularly if the expressed proteins may find strong binding partners within the cell. But it could work well if the isolated proteins are carefully washed in high salt, etc.

A2: We agree that reconstituting HR fragments linked to the transmembrane domain (TMD) of Mitofusin is really interesting and, indeed, something that we initially planned to do. However, in our homotypic fusion system, HR1 domains must be added at t=0 of the FRET-based lipid mixing assay in order to induce fusion. Nevertheless, we have tested the hypothesis of the reviewer by a similar approach: we coupled HR1 to maleimide lipids in the presence of detergents (generating a molecule similar to the proposed HR1-TMD) and then reconstituted it into liposomes by detergent dilution/dialysis. However, when the fusion assay was performed, no lipid mixing was measured between these HR1-containing liposomes (Fig. R2), which we attributed to the fact that fusion had already occurred during proteoliposomes reconstitution and storage. Purifying a TMD anchored HR1 fragment would thus not help in the context of these homotypic fusion experiments because we would not be able to add such construct for direct insertion into liposomes at the beginning of the FRET assay (the presence of detergent in the purification buffer would destabilize the liposomes). But, it is in fact problematic that we could not vary the lipid composition in the homotypic/symmetrical liposome fusion system when using maleimide lipid anchorage. To overcome this issue, we decided to develop a new anchoring strategy using a lipid whose headgroup is functionalized with an NTA-Ni complex and to synthesize an HR1 fragment with a C-terminal His_6 tag that forms coordination bonds with Ni^{2+}. This new HR1-His_6 fragment could induce efficient lipid mixing between POPC:NTA-Ni (95:5) liposomes (Fig. R3). Importantly, the NTA-Ni anchoring strategies also allowed us to vary the lipid composition of the liposomes without preventing coupling of HR1-His_6 to the liposome membrane. Addition of 30 mol% of DOPE lipids dramatically increased the extent of lipid mixing, as expected by the higher affinity of amphipathic helices for membranes displaying lipid packing defects (Fig. R3). More lipid compositions were tested as suggested by reviewer 3, and the corresponding results are presented later in this response.

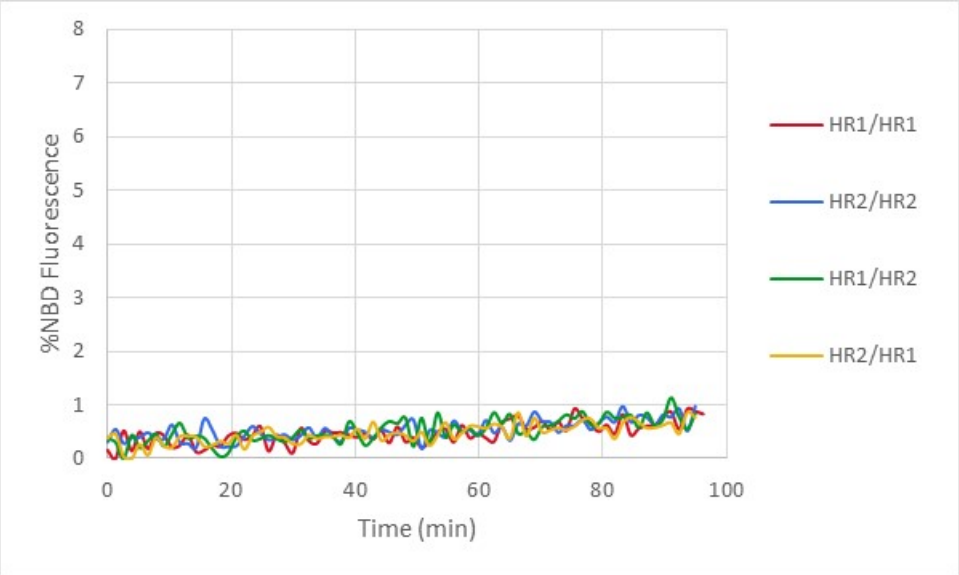

Figure R2. FRET-based lipid mixing experiments between POPC:C45 (95:5) liposomes reconstituted with HR1 or HR2 fragments (using co-micellization of lipids and HR fragments pre-coupled to maleimide lipids followed by dilution/dialysis) prior to running the fusion assay. No fusion was measured for all combinations tested (legend: HRx/HRy = non-fluorescent liposomes of HRx vs. fluorescent liposomes of HRy).
Data not shown in the new manuscript

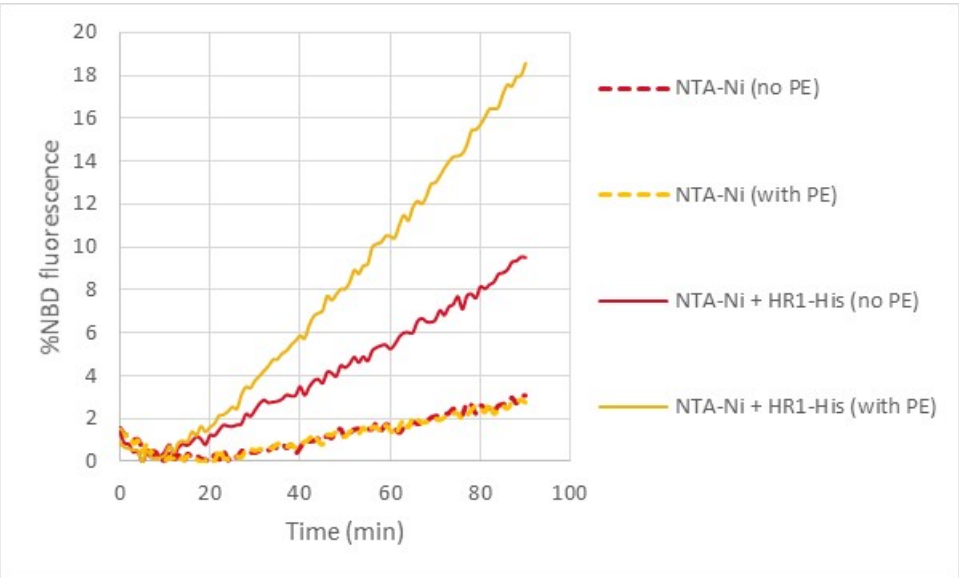

Figure R3. FRET-based lipid mixing experiments between POPC:NTA-Ni (95:5) or between POPC:DOPE:NTA-Ni (65:30:5) liposomes in the absence or presence of HR1-His_6 added at t=0 of the assay. The HR1-His_6 fragment induces efficient lipid mixing between liposomes and fusion is activated when the liposome membrane contains 30 mol% DOPE lipids.
Data of this figure is shown in Figure S12A and described on page 14 of the new manuscript

Q3: There is an essential control that should be done throughout the study - which is to mix HR1 with HR2 containing liposomes. There are many who would argue that these should also be able to bind in trans (notably the recent study by the Dorn lab PMID:27775718), so this pairing should be included.

A3: We explored various ways to perform such an experiment. The first one consisted in mixing liposomes reconstituted with either HR1 or HR2 using the co-micellization/dialysis method. No fusion was measured between HR1 and HR2 liposomes by this approach (Fig. R2). However, since HR1 was also incapable of inducing fusion when proteoliposomes were formed prior to running the

fusion assay (Fig. R2), we needed to find an alternative strategy. The main difficulty was to find an approach that would allow us to couple HR1 and HR2 to two separate liposome preparations and would still be compatible with initiation of fusion at t=0 of the assay. To do this, we used the 3 following alternative approaches:

a) Pre-incubation of fluorescent maleimide-containing liposomes for 1 hour at 37°C with HR2 and addition of these HR2 liposomes to non-fluorescent Maleimide-containing liposomes at t=0 of the assay together with HR1 (because the volume of fluorescent liposomes added in the FRET assay is 8 times less than that of non-fluorescent liposomes – see method section for details – any uncoupled HR2 proteins are largely diluted and do not interfere with HR1 coupling).
b) Overnight pre-incubation on ice of non-fluorescent and fluorescent maleimide-containing liposomes with HR1 and HR2, respectively (we have previously checked that no fusion occurs on ice) and posterior mixing the resulting HR1 and HR2 liposomes at t=0 of the FRET assay.
c) Using a double anchoring strategy that is now allowed by NTA-Ni lipids: addition at t=0 of the FRET assay of HR2 and HR1 bearing respectively an N-terminal Cysteine tag (Cys-HR2) and a C-terminal His_6 tag (HR1-His_6) to non-fluorescent maleimide-containing liposomes and fluorescent NTA-Ni-containing liposomes, respectively (or similarly addition of HR1-His_6 and Cys-HR2 to non- fluorescent NTA-Ni liposomes and fluorescent maleimide liposomes, respectively).

We display here the results from approach "c" (Fig. R4). All three approaches gave the same results: a reduction of 40-60% of the extent of fusion in the HR1/HR2 system compared to the HR1/HR1 system. Importantly, a similar reduction was observed when HR1 liposomes were fused with protein-free liposomes (Fig. R4), indicating that the measured fusion activity was due to HR1/membrane interaction rather than HR1/HR2 interaction in trans (across bilayers).

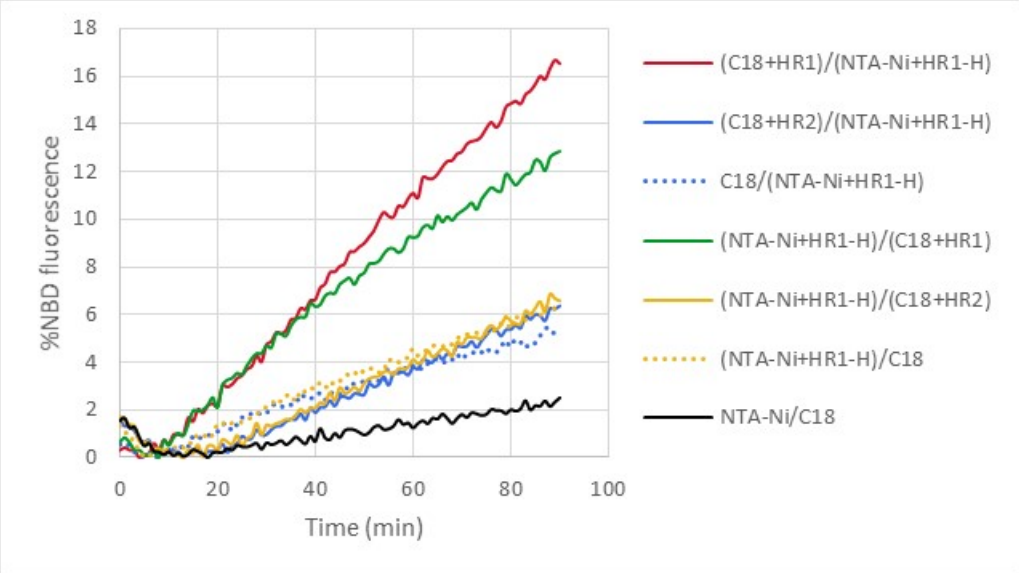

Figure R4. FRET-based lipid mixing experiments between POPC:C18 (95:5) and POPC:NTA-Ni (95:5) liposomes in the presence of HR1 (with a C-terminal Cysteine or His_6 tag) and HR2 (with an N-terminal Cysteine tag) added (in various combinations) at t=0 of the assay. Similar fusion extents were measured between HR1 liposomes and protein-free or HR2 liposomes, indicating that fusion is due to HR1 interacting with the liposome membrane and not with HR2.
Data of this figure is shown in Figure S12B and described on page 14 of the new manuscript

Additional experiments performed in solution confirmed that HR1 and HR2 do not strongly interact in our system. Notably, circular dichroism (CD) experiments revealed no change in the mean residue molar ellipticity at 222 nm of a mixture of HR1 and HR2 compared to a theoretical signal built from the isolated signals of HR1 and HR2, assuming no interaction between them (Fig. R5).

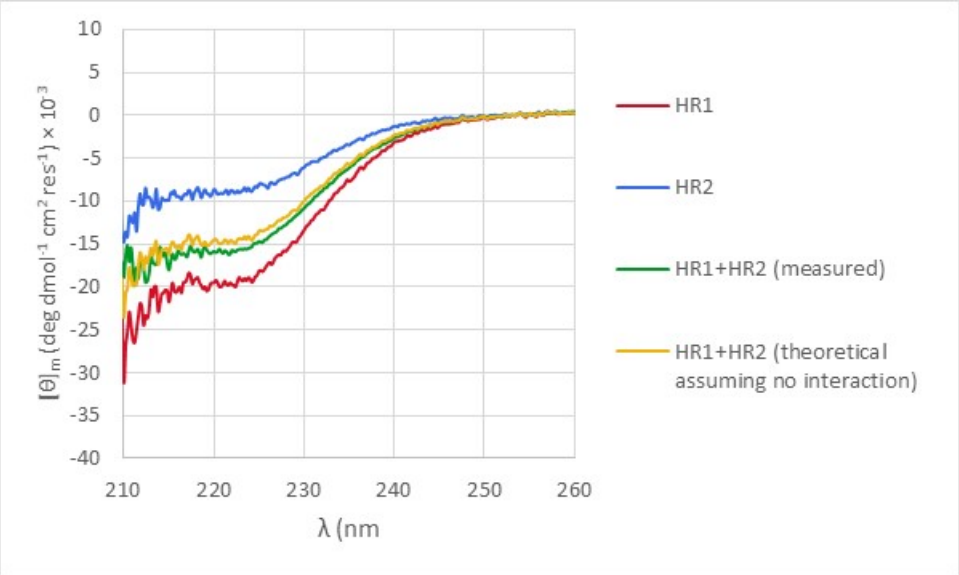

Figure R5. Circular dichroism spectra of HR1 and HR2, together or isolated, after 20 min of incubation at 37°C at a final concentration of 20 µM (these spectra were recorded in HEPES buffer, which explains the noise measured below 210 nm).

Data not shown in the new manuscript

The absence of any measurable HR1/H2 in our system is maybe not surprising given that previously reported HR1/HR2 interactions occurred with HR1 or HR2 within the framework of a folded Mitofusin protein (Rojo et al, J Cell Sci, 2002; Huang et al, PLOS ONE, 2009; Franco et al, Nature, 2016). In line with this, an isolated C-terminal Mfn1 fragment (residues 629-741) failed to dimerize (Qi et al, J Cell Biol, 2016) although it included the HR2 domain that was proposed to form homotypic trans-interactions across mitochondria.

Q4: It is not clear what the protein/lipid ratio is for the cysteine-HR1/2 added to the liposomes for fusion or tethering. It is important to include a titration of HR1/2 to determine the stoichiometry that may drive fusion and whether this makes sense with endogenous expression within mitochondria. Previous experiments with Atlastin-reconstituted liposomes included these kinds of experiments, which were very informative (Orso et al. 2009; Lee et al. 2015)

A4: We agree that the minimal protein surface density allowing liposome fusion is an important parameter that was missing in our manuscript. So we have performed additional lipid mixing experiments with different concentrations of HR1 added at t=0 of the assay and quantified the actual lipid-to-protein ratio of each liposome preparation in a separate liposome co-floatation assay (Fig. R6). This new set of experiments revealed that significant lipid mixing (larger than 5% after 90 min of reaction, that is 2 fold higher than the fusion background of the protein-free control) required at least 1 HR1 protein for 470 lipids (obtained by the addition of 3.125 µM HR1 at the beginning of the fusion assay). Of note, addition of 12.5 µM of HR1, as performed throughout the manuscript, leads to a lipid-to-HR1 ratio of 130.

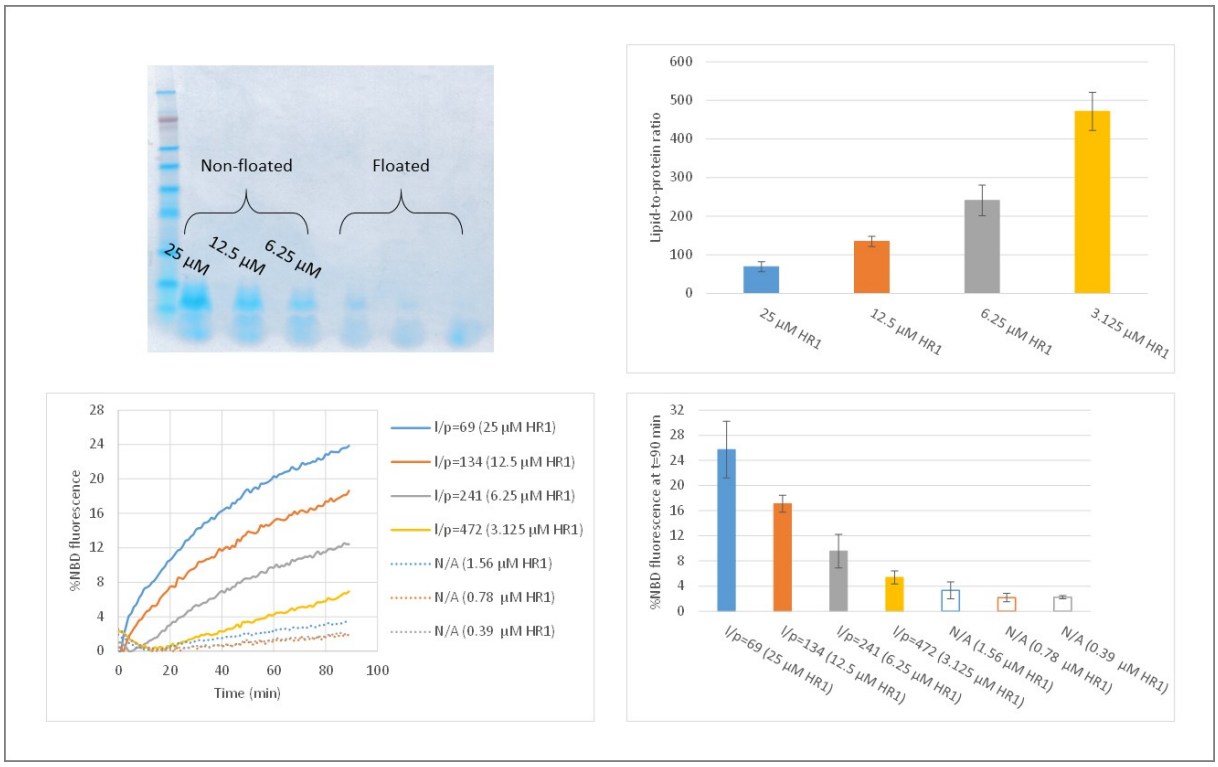

Figure R6. Liposome fusion activity as a function of the surface density of HR1 on the liposome membrane. Different concentrations of HR1 were added at t=0 of the FRET assay (from 25 µM down to 0.39 µM final). The actual lipid-to-protein ratio was quantified in a separate experiment by floatation of the liposomes on a Nycodenz gradient. Protein and lipid recoveries were measured by SDS PAGE stained with Coomassie upon comparison with the non-floated liposome samples. Silver staining was used when the peptide was not visible by Coomassie staining. The protein concentration in the floated proteoliposomes resulting from incubations with HR1 at 1.56 µM or less could not be measured (detection limit of silver staining for these peptides).
Data of this figure is shown in Figures S5A & S5B and described on page 9 of the new manuscript

How does these densities compare with known surface densities of fusion proteins, such as SNARE and Atlastin proteins, as well as with the physiological concentration of Mitofusins in the outer mitochondrial membrane? The concentration of the v-SNARE protein VAMP2 in synaptic vesicles was shown to be about 12,600 molecules/µm^2 (Takamori et al, Cell, 2006), whereas the average concentration of the t-SNARE protein Syntaxin 1 at the plasma membrane was estimated to be of 1800 molecules/µm^2 with active Syntaxin 1 molecules concentrating in small domains (of 50-60 nm) where their surface density can be as high as 31,600 molecules/µm^2 (Sieber et al, Science, 2007). In the context of liposomes (assuming 0.65 nm^2 per lipid), this would correspond to a lipid-to-VAMP2 ratio of 120 and a lipid-to-Syntaxin ratio between 50 and 800. We have previously shown that efficient *in vitro* SNARE-mediated liposome fusion (>5% after 80 min of reaction) requires that both v- and t-SNARE proteins have surface densities of at least 1 protein for 300 lipids (Ji et al, Biophys J, 2010). Similar protein-to-lipid ratio was required for efficient Atlastin-mediated liposome fusion *in vitro* (Orso et al, Nature, 2009; Anwar et al, J Cell Biol 2012). By using liposomes with a lipid composition mimicking that of the ER, less Sey 1p (the yeast homolog of Atlastin) was required and fusion was efficient at a lipid-to-protein ratio of 1000 (Lee et al, J Cell Biol 2015). The SNARE and Atlastin protein surface densities required for liposome fusion are thus comparable with those allowing fusion by HR1 in our system.
How does it compare with the physiological concentration of Mitofusins? To answer this question, we have purified mitochondria from wild-type MEF cells and quantified by Western Blot their Mitofusin content by comparison with several concentrations of recombinant MBP-Mfn1 and MBP-Mfn2. By this approach, we measured 0.22 ng of Mfn1 and 0.74 ng of Mfn2 per µg of mitochondria (Fig. R7). By modelling mitochondria as an ellipsoid of 1 µm * 1 µm * 10 µm with a density of 1.19 g/cm^3 (J.M. Graham and D. Rickwood, Subcellular Fractionation: A Practical Approach, Oxford University Press, 1997), this leads to 798 Mfn1/µm^2 and 2602 Mfn2/µm^2. In the context of

liposomes, this would correspond to lipid-to-protein ratios of 1927 and 591, respectively, and thus to a total lipid-to-Mitofusin ratio of 452. The total concentration of Mitofusin is thus comparable to the lipid-to-protein ratio required for fusion in our *in vitro* system, but the Mfn1 concentration is slightly lower. *In vivo*, Mfn1 could locally concentrate at sites of mitochondrial fusion as suggested by the recent cryo-EM work of the Cohen lab, which showed that mitochondrial fusion occurred at the edge of a docking ring containing high protein densities (Brandt et al, Elife, 2016).

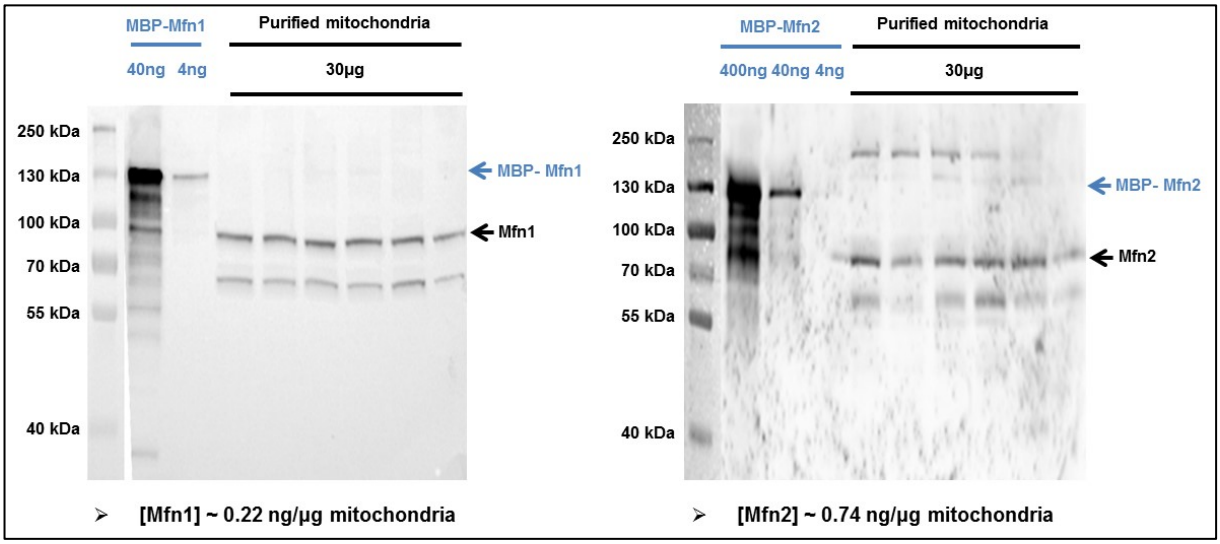

Figure R7.
Data of this figure is shown in Figure S5C and described in the Supplementary Text of the new manuscript

Q5: The authors should really attempt to examine the size of any complexes that may form between the C18 and C45 conjugated peptide. Do they stay as monomers or assemble into larger structures? These could be examined by BN-PAGE or sizing columns. Early work has established distinct complexes in cis vs trans (PMID:15572413), and more recent work by the Cohen lab has shown the large ring structures (that the authors cite in the discussion). It should be straightforward to test the types of assemblies formed by the HR1 vs HR2 conjugates in the bilayer.

A5: To examine possible oligomerization of HR1 or HR2 within lipid bilayers, we first thought of testing the SDS resistance of such potential oligomeric HR complexes (as is the case for the coiled-coil structure formed by the HR regions of t-SNARE and v-SNARE proteins). HR1 or HR2 were incubated for 1 hour at 37°C with Maleimide-containing liposomes and these liposomes (non-floated or floated on a nycodenz gradient) were analyzed by SDS PAGE, following or not warming-up of the samples for 10 min at 70°C (Fig. R8, top). In all cases, we did not observe on the gel any visible large molecular weight complex indicating that HR1 or HR2 did not form any SDS resistant homotypic complex within the liposome membrane (similar results were obtained in experiments performed in solution). Next, we therefore tried to examine complexes by Native PAGE, which turned out to be very complicated due to the small size of the peptides and the presence of lipids in the sample. After many trials and errors (varying the peptide concentration, the staining and loading conditions, the nature of detergent, *etc.*), we found that the best results were obtained with Invitrogen NativePAGE Bis-Tris gels in the presence of a very low (0.1%) amount of n-dodecyl-β-D-maltoside (DDM) detergent. All samples did migrate as a single band on the gel, indicating that HR1 and HR2 did not form any large oligomeric structure at the liposome bilayer surface even under non denaturing conditions. Because these gels could not resolve complexes less than 15 kDa, we however cannot exclude that HR1 or HR2 may form dimers in cis. These results suggest that cis-oligomerization of Mitofusin observed in previous reports (Ishihara et al, J Cell Sci, 2004; Brandt et al, Elife, 2016) involves other functional regions of the protein, very likely its GTPase domain as indicated by the GTP-dependent oligomerization state of Mfn1 (Ishihara et al, J Cell Sci, 2004), the recent crystal structure of the GTPase domain linked to HR2 *via* a flexible linker (Qi et al, J Cell Biol, 2016; Cao et al, Nature, 21017), and the observation that formation of the Mitofusin docking ring requires GTP hydrolysis (Brandt et al, Elife, 2016).

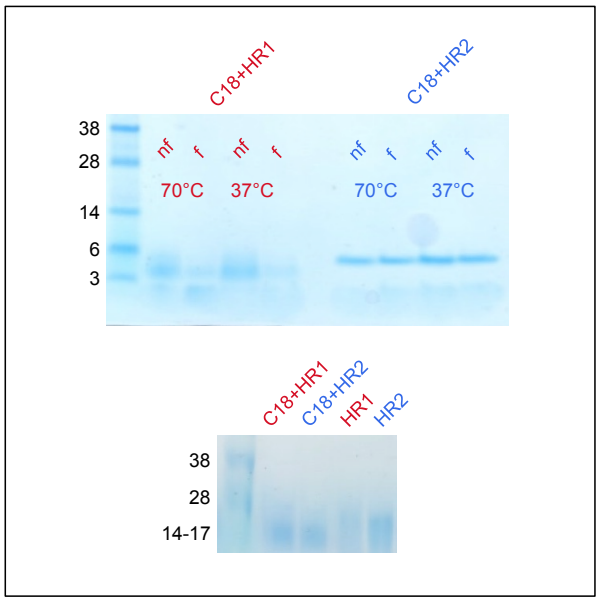

Figure R8. Top, SDS PAGE analysis of HR1 and HR2 liposomes (formed by incubating 500 µM of lipids with 12.5 µM of proteins for 1 hour at 37°C) before and after floatation on a nycodenz gradient. Samples were incubated or not for 10 min at 70°C prior to loading on the gel to disrupt any potential SDS resistant complexes. Bottom, Native PAGE analysis of floated HR1 and HR2 liposomes (prepared as above) and comparison with the signal of HR1 and HR2 proteins (both at 12.5 µM).

Data not shown in the new manuscript

Q6: The fact that HR1 mediates lipid mixing with both C18 and C45 anchored HR1 is intriguing, and to this reviewer suggests that hemifusion is the primary mode of action. In eliminating fluorescence of outer leaflet, the authors revealed 40% of liposomes underwent full fusion while 60% only hemifusion. Again, I think that kinetic analysis of fusion in this quenched outer leaflet mode may be helpful if, for example, HR1 anchors the lipids to initiate hemifusion, which may spontaneously resolve into full fusion after a certain time. This is distinct from the SNAREs (as the authors explain), which only fuse C45 anchored liposomes.

A6: We agree that HR1 interaction with the outer lipid monolayer very likely mediates hemifusion which then spontaneously transits to full fusion. To get further insight into the mode of action of HR1 in fusion, we have analyzed the percentage of hemifusion at different time points of the inner leaflet lipid mixing assay. This kinetic analysis revealed that the percentage of hemifusion events was slowly and continuously decreasing as a function of time (Fig. R9), suggesting that liposomes were constantly going through hemifusion and full fusion events during the experiment, and thus did not accumulate for a long time (at least not larger than the time resolution of the FRET assay, which is 1 min here) in hemifused states before progressing to full fusion. In an attempt to favor/stabilize hemifusion events, we have also performed lipid mixing experiments with respectively two times more (25 µM) and two times less (6.25 µM) HR1 added at t=0 (leading to actual lipid-to-protein ratio of ~70 and 240, respectively) but similar results were obtained, with a percentage of hemifusion that remained within 60-80% over the course of the experiment. With even less HR1 added (≤3.125 µM), fusion was too low to allow analysis before/after sodium dithionite treatment.

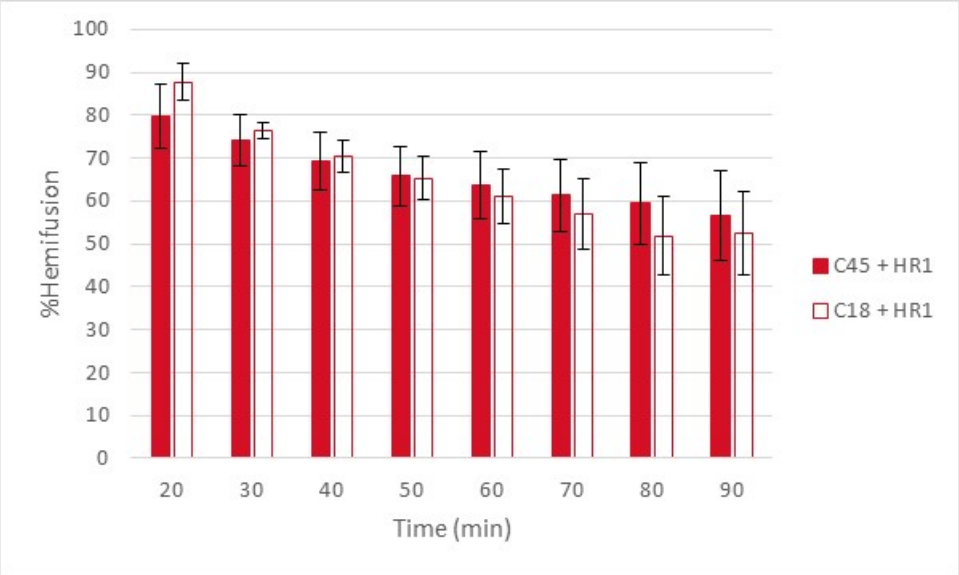

Figure R9.
Data not shown in the new manuscript

Q7: Related to that last comment, if kinetic and/or titrations were performed it could be possible to capture the hemifused states by EM. Currently this is inferred from the fluorescence and I think it could provide an important mechanistic advance.

A7: This is a good idea but this is also very challenging. To maximize our chances of capturing hemifusion events by cryo-EM, we first tried to find experimental conditions that could favor/stabilize hemifusion in the FRET assay, but neither changing the HR1 concentration nor measuring FRET at different time points of the assay appeared to facilitate observation of hemifusion events. So we decided to keep the same experimental conditions as in the fusion assays performed throughout the manuscript, and to observe liposomes by cryo-EM after 1 hour of incubation at 37°C between 500 µM of lipids and 12.5 µM of HR1 (leading to an actual lipid-to-protein ratio of ~130). On the obtained cryo-EM pictures, we could often see liposomes in very close apposition without being able to tell for sure whether they were mixing their outer monolayers (Fig. R10, left; events indicated by arrows), and we occasionally captured structures (Fig. R10, right) that closely resembled hemifusion intermediates induced by HA proteins (Chlanda et al, Nat Microbiol, 2016). These preliminary results indicate that hemifusion structures are very difficult to capture, most likely because they are transient structures that quickly resolve into full fusion in our system. We also noted that it was difficult to clearly distinguish the two lipid monolayers in the case of small liposomes and that it became easier when the diameter of the liposomes was 100 nm or more. Because large liposomes are less prone to fuse in the presence of HR1 (except in the presence of PE lipids), one possibility for future experiments would be to perform cryo-EM with 100 nm NTA-Ni liposomes of various compositions (*e.g.* including PE, CL or PA lipids). Because PE lipids are known to stabilize the hemifusion structure, this could also facilitate observation of this intermediate. We believe that it is too early to include these cryo-EM data in our manuscript and we would prefer to first explore things further.

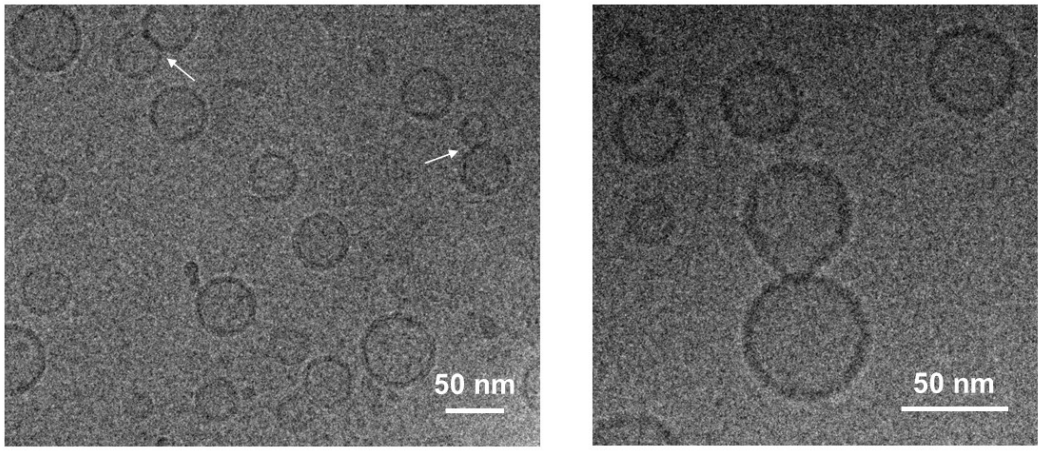

Figure R10.
Data not shown in the new manuscript

Q8: The authors tested whether HR1 and HR2 can tether liposomes upon incubating biotinylated, non-fluorescent liposomes with fluorescent liposomes, in the presence of streptavidin beads, followed by fluorescence that is pulled down with the beads (Fig4A). The results are not very convincing, with large error bars indicating low significance, although the incubation was done overnight. The inclusion of SNARE pairs as a positive control is very important, but again, a kinetic and titration analysis would really help to understand the physiological significance of this tethering. Very early work by Mihara's group suggested that Mfn1 but not Mfn2 could tether mitochondria, and that the hydrolysis and exchange rates of Mfn1 were much higher than Mfn2 (PMID:15572413). The GTP dependence of the tethering shown back then is consistent with the model emerging from the crystal structure that the tethering is done through the head to head binding of the GTPase domains in trans. From all of this, I'm not convinced that the HR domains would really be efficient tethers. Therefore, it is important to expand this figure with additional kinetic analysis.

A8: The error bars are in fact large, notably for SNARE liposomes because we had variability in the efficiency of SNARE reconstitution in these experiments. This is much better for experiments performed with HR1 and HR2 and, importantly, fluorescence values for HR and SNARE liposomes are always much higher than for protein-free liposomes. We believe that it is reasonable to say that we have about 20-30% of docking in all cases, and this result is consistent with that obtained by negative stain EM. Overnight incubation on ice is not ideal (notably not physiological), but we did it to prevent liposome fusion by HR1. To strengthen these data and better understand the mode of action of HR1 and HR2 in liposome docking, we have performed several additional experiments by Dynamic Light Scattering (DLS). The kinetics of liposome docking by HR2 was monitored for 1 hour at 37°C with one measurement every 5 min under the same experimental conditions as in the FRET assay (500 µM lipids and 12.5 µM peptide). The liposome size distribution started shifting toward larger values (due to docking since no HR2-mediated liposome fusion was measured in the FRET assay) after 10-15 min, and the liposome size increased continuously during the reaction (Fig. R11, top left). Studying docking of liposomes by HR1 at 37°C is complicated by the occurrence of liposome fusion in this case (the increase of particle size can be due to docking and/or fusion). To overcome this issue, we have used larger liposomes (100 nm) prepared by extrusion. Surprisingly, no liposome docking was observed by HR1 in this system, whereas HR2 could still dock these larger liposomes (Fig. R11, top right). This suggests that docking by HR1 as previously observed (Fig. R11, bottom left) was essentially due to the interaction of HR1 with the membrane of small liposomes (and not HR1/HR1 interaction). This interaction, which vanishes when the membranes are not curved enough, is also expected to require the amphipathic helix of HR1. To confirm this, we have monitored the capacity of an amphipathic helix deleted mutant peptide (HR1-aH) to mediate the docking of small liposomes. As expected, this mutant could not induce liposome docking (Fig. R11, bottom right). We thank the reviewer for this suggestion that allowed us to better understand the mode of action of HR1 in the observed liposome docking events.

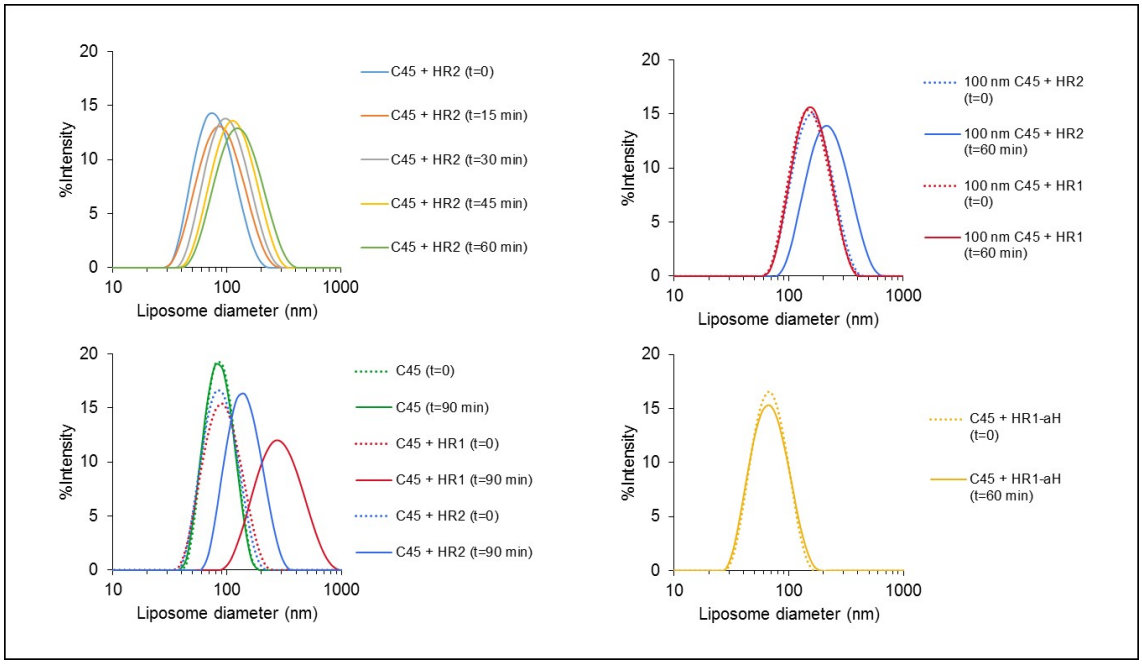

Figure R11.
Data of this figure is shown in Figure S7 and described on page 15 of the new manuscript

Q9: The authors examined the tethering mediated by HR1/2 by EM. However, it isn't clear how "clustering" is defined in terms of nm spacing between membranes. The field has a concept of 20-30nm as a distance between opposing membranes that are functionally tethered. Are these tethers dilution resistant? Perhaps I missed that point.

A9: We realize that this requires clarification in the manuscript since the definition of tethered and docked states may vary across labs. Throughout the manuscript, we referred to docking rather than tethering in order to directly compare with the mode of action of SNARE proteins in vesicular transport. During intracellular vesicle trafficking, membranes are first anchored/tethered to each other at distances of several tens of nanometers by Rab GTPase proteins and tethering factors; membranes then come into close proximity (dock to each other at distances lower than 10 nm) when cognate v- and t-SNARE proteins assemble in the form of a parallel coiled-coil complex of four HR domains. In the context of these definitions, the proposed trans-dimerization of Mfn1 through its GTPase domain (Ishihara et al, J Cell Sci, 2004; Qi, Y. et al, J Cell Biol, 2016; Cao et al, Nature, 2017) could induce mitochondrial tethering (at ~30 nm from each other), and the trans-dimerization of HR2 domains could occur subsequently to mediate mitochondrial docking (at <10 nm from each other). We have now specified in the legend of Fig. 4B what distance criteria was used to define docking in our study. In the EM experiments shown in Fig. 4B, we considered that two liposomes were docked to each other when their membranes were separated by 5 nm or less. Note that this definition might lead to an underestimation of the number of docking events since the crystallized antiparallel HR2 dimer is ~10 nm long (Koshiba et al, Science, 2004). EM pictures were taken on samples that had been diluted 20 times following the 1 hour incubation period at 37°C. This information was given in the method section of the manuscript; it is now also indicated in the legend of Fig. 4B.

Referee #2:

Authors examined the biochemical/biophysical properties of the HR1 domain of mitofusin, which is important for mitochondrial outer membrane fusion and for maintenance of mitochondrial function. They show that (i) HR1 domain is required for mitofusin-mediated mitochondrial fusion in cultured cells, (ii) HR1 domain induces membrane fusion and docking using in vitro fusion assay, although HR2 only mediates docking, (iii) membrane-anchored HR1 induced heterotypic fusion with protein-free liposome, (iv) membrane binding and perturbation properties of HR1 are from its amphipathic

helix structure. The authors propose a model in which HR1 interacts with the lipid membrane and then, brings membrane in close and perturbs the lipid bilayer structure to drive membrane fusion.

The subject of this study is highly relevant and interesting because little is known about the mechanism of membrane fusion by mitofusin proteins. Observing membrane fusion and docking with HR1 peptide is partly significant, however, this study add less to molecular understanding of mitochondrial fusion, since this study is very limited in HR1 domain, and these data are just descriptive.

Critical issues in this manuscript are:

Q1: The membrane-destabilizing property of HR1 is speculative - no direct experimental evidence is provided.

A1: For fusion to occur, membranes must transit from a bilayer to a non-bilayer structure, which is a perturbation/destabilization of the membrane. In this regard, we have therefore directly shown that HR1 can perturb membranes. We have also shown that this effect can be modulated by changing the packing properties of lipid bilayers (this was accomplished by modifying either their curvature or their lipid composition), a property that is directly related to bilayer stability. New fusion experiments performed with an amphipathic helix deleted mutant peptide now also show that both the docking and fusion activities of HR1 require its amphipathic helix (aH). Docking occurs when the aH binds to the opposite membrane and fusion when it folds in contact with this membrane (and thus perturbs its structure). For more direct observation of changes in the biophysical properties of membranes by HR1, we are currently exploring structural approaches such as cryo-EM and 31P NMR. Preliminary cryo-EM pictures allowed us to directly observe a hemifusion structure induced by HR1 (Fig. R10). 31P NMR will allow us to evaluate the extent of bilayer perturbation by HR1. These approaches will be the object of a future study. Because we do not show any structural data on bilayers in the present manuscript, we have softened our claim in the abstract and replaced “Our results show that HR1 facilitates membrane fusion by destabilizing the lipid bilayer structure (…)” with “Our results strongly suggest that HR1 facilitates membrane fusion by destabilizing the lipid bilayer structure (…)”.

Q2: Significance of the HR1 mediated liposome docking by homotypic and heterotypic in Mfn1-mediated mitochondrial fusion are not clear.

A2: A new series of experiments performed by Dynamic Light Scattering with larger liposomes, as well as experiments using a new synthetic HR1 fragment lacking its 18-residue amphipathic helix, have allowed us to better understand the mode of action of HR1 in liposome docking. These experiments showed that the observed docking activity of HR1 was essentially due to HR1/membrane interaction through its amphipathic helix and not to HR1/HR1 interaction (see answer to question 8 of referee #1 and Fig. R11).

Other points:

Q3: It is not clear how much protein was used for experiments in Fig.2-7. I did not find such important information in main text.

A3: This information was given in the method section but was in fact missing from the figure legends. This is now indicated and, more importantly, we have now estimated the actual protein-to-lipid ratio after reconstitution into liposomes. This allowed us to identify the minimal HR1 surface density that is required to mediate liposome fusion (see answer to question 4 of referee #1 and Fig. R6).

Q4: It is not clear why both synthetic and recombinant HR1 are used in this study.

A4: All pilot experiments of this study were performed with recombinant HR1 and HR2 fragments expressed in *E. coli*. Because we were concerned that the observed fusogenic activity of HR1 fragments could be due to a possible bacterial contaminant, we decided to also perform experiments with synthetic peptides of very high purity. These synthetic fragments behaved qualitatively as the recombinant fragments, and gave reproducible and consistent results. In the submitted manuscript,

experiments with synthetic HR1 and HR2 are presented in the main figures, and experiments with recombinant HR1 and HR2 expressed in *E. coli* are presented in Figs. S4 and S8. Except otherwise noted in the figure legends, HR1 and HR2 fragments used in this study were therefore synthetic peptides.

Referee #3:

The authors present a very nice dissection of the promotion of membrane fusion by MFN1 HR1.

Specific comments:

Q1: Fig. 4B - it would be good to have a picture here of the C45 image, which is the negative control, in addition to the two experimental conditions, +HR1 and +HR2. It's not possible to interpret the significance of the docked and enlarged vesicles without having the starting conditions / negative control to compare them to.

A1: We agree that it is important to also show a representative picture of the statistics obtained with protein-free C45 liposome. So we have added a new panel in Fig. 4B showing protein-free C45 liposomes after 1 hour of incubation at 37°C.

Q2: Fig. 7B - negative results, in particular, require validation of the experimental details, to attempt to rule out as many trivial explanations as possible. In this instance, the authors show that excision of the 18-amino acid amphipathic helix eliminates the fusion promoting capacity of Mfn1. However, there is no validation of this construct, e.g. is it expressed at similar levels (western blotting) and does it subcellularly localize correctly (microscopy)? Although the findings are consistent with the proposed model, they could be strengthened as described above and using other approaches (e.g. the assays used in prior figures).

A2: This concern was echoed in the report of referee #1. To rule out that the observed phenotypes could be due to a default in protein expression level and/or a lack of protein localization at mitochondrial membranes, we have performed western blotting, co-localization experiments by immunofluorescence microscopy, and protein extraction experiments from purified mitochondria. Western blotting revealed that all constructs were expressed at similar levels in Mfn1 KO MEFs (Fig. R1, top left), and microscopy experiments showed that they were all strongly co-localizing with an EGFP expressed in the mitochondrial matrix (Fig. R1, top right). In addition, preliminary biochemical experiments on purified mitochondria indicated that all Mfn1 variants were inserted into the outer mitochondrial membrane *via* their transmembrane domain (Fig. R1, bottom). To strengthen these results, we have also synthetized a new HR1 mutant peptide lacking the 18-residue amphipathic helix (HR1-aH), and performed *in vitro* liposome docking and fusion experiments with this mutant. The HR1-aH variant could not induce either liposome docking (Fig. R11, bottom right) or liposome fusion (Fig. R12) in the DLS and FRET assays, respectively. These results confirmed the importance of the amphipathic helix of HR1 for fusion and revealed that liposome docking by HR1 was essentially due to HR1/membrane interaction through its 18-residue amphipathic helix.

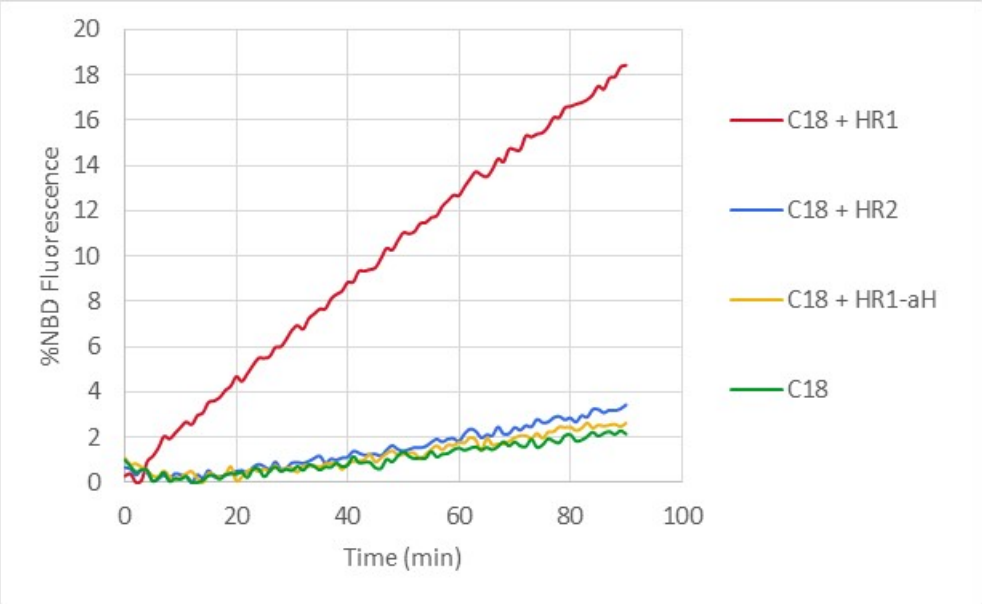

Figure R12.
Data of this figure is shown in Figure S11B and described on page 15 of the new manuscript

Q3: More generally, while the work is carefully performed using PC liposomes as described, PC liposomes are far from physiological. This is evidenced by the rescue of fusion using PE with larger liposomes for HR1. One wonders how HR2 might behave in this assay, or the mutant lacking the amphipathic helix. Some of the other lipids reported to be present in high quantities in contact sites, such as CL (up to 25% of the membrane lipids there) or phosphatidic acid (another cone shaped lipid) are thought to have profound effects on membrane structure and order. Whether the well-described findings here would remain equally true with more physiological membrane targets seems unknowable without testing (e.g., whether HR2 would exhibit fusogenic activity).

A3: Working with liposomes composed exclusively of PC lipids is in fact far from physiological but however presents the advantage of not favoring fusion due to the cylindrical shape of these lipids (in contrast to *e.g.* PE lipids that are cone-shaped and known to facilitate transitions from bilayer to non-bilayer structures). That said, having now fully characterized the fusion activity of HR1 with PC liposomes, we agree that it was important to investigate the effect of other lipids found in the outer mitochondrial membrane. In the manuscript, using the asymmetrical fusion system, we have shown that PE lipids could restore fusion by HR1 when the membrane curvature became a limiting factor. These experiments were also performed with HR2 and revealed that HR2 did not present any fusogenic activity even when 30% PE was included in the target membrane (Fig. R13, top left). In addition to PE lipids, the outer mitochondrial membrane contains CL and PA lipids that are also known to regulate mitochondrial fusion (Frohman, J Mol Med, 2015). CL lipids are present at about 5 mol% in the outer mitochondrial membrane but their local concentration at sites of contact between the outer and inner mitochondrial membranes can reach up to 20-25 mol% (Ardai et al, J Biol Chem, 1990). We therefore tested the effect of two concentrations of CL (5 and 20%) in HR1-mediated liposome fusion. For PA lipids, since cleavage of 1 CL molecule leads to 2 PA lipids, we arbitrarily chose to test the effect of 10 mol% PA in our fusion assay. This new set of experiments revealed that 5% CL or 10% PA had no clear effect in the asymmetrical fusion system with 100 nm liposomes, whereas 20% CL had a net activatory effect, approaching that observed with 30% PE (Fig. R13, top right). HR2 and the amphipathic helix deleted HR1 mutant (HR1-aH) could still not induce liposome fusion for all lipid compositions tested (Fig. 13, bottom left and right). We also took advantage of a new anchoring strategy using NTA-Ni lipids to investigate the effect of PE, CL and PA lipids in the symmetrical fusion system. Addition of 30% PE strongly activated fusion by an HR1 fragment containing a C-terminal His_6 tag (Fig. R14), in agreement with the observations made in the asymmetrical system. In contrast, CL and PA both appeared to have a slightly inhibitory effect in this system (Fig. R14). These different behaviors could be explained by possible interactions between HR1 and CL lipids that can occur only in trans in the asymmetrical system, but both in cis and trans in the symmetrical system. This will require further investigation, taking into account the size of liposomes and the surface densities of proteins with the different lipid anchors.

Because we cannot currently explain the results obtained with CL and PA lipids, we prefer not to include them in the present manuscript.

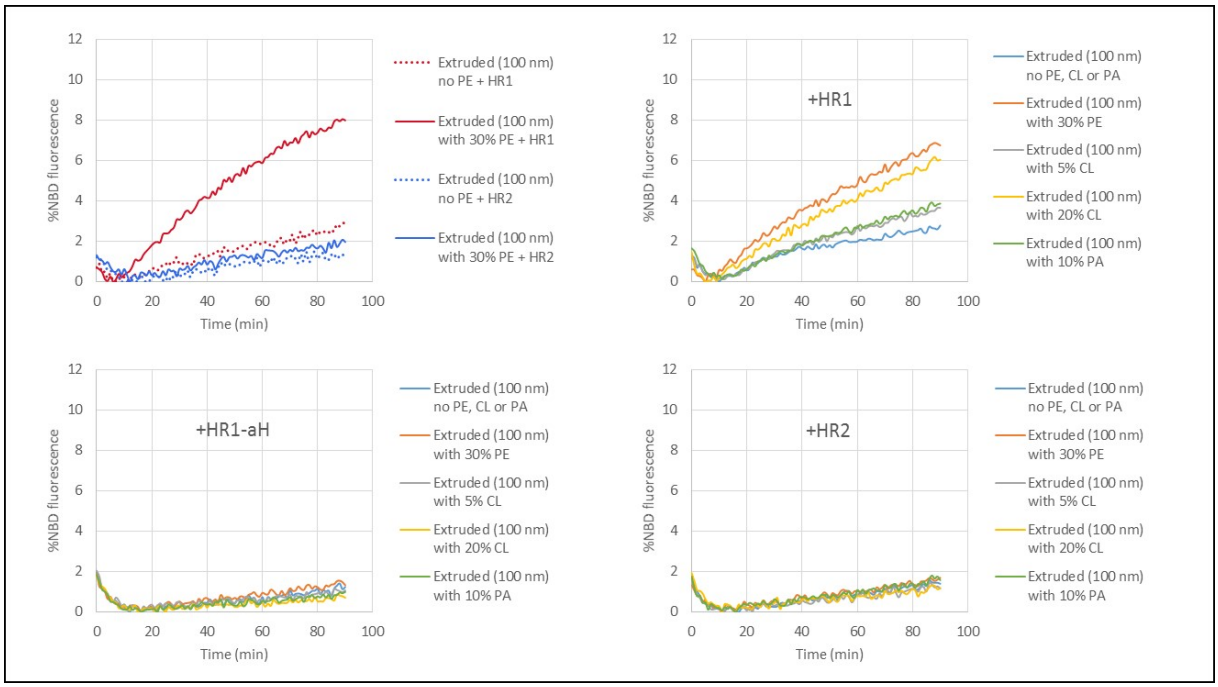

Figure R13.
Data of this figure (top left panel) is shown in Figure S11A of the new manuscript

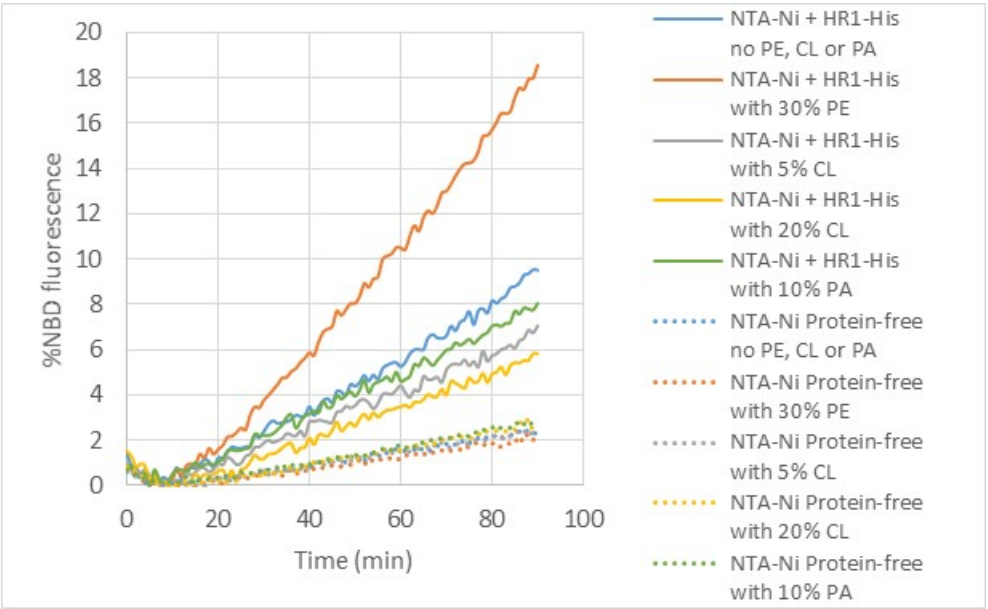

Figure R14.
Data not shown in the new manuscript

Q4: Is there a cognate amphipathic helix in HR2?

A4: This is an interesting question that requires to first restate how we postulated and found the amphipathic helix (aH) within HR1. Using Heliquest (http://heliquest.ipmc.cnrs.fr/), we searched for the presence of an aH within the HR1 sequence using a sliding window of 18 residues. We obtained the highest hydrophobic moment towards the end of HR1 sequence (393-DVKKKIKEVTEEVANKVS-410), which made us postulate the existence of an aH there. Interestingly, this is exactly at this position that the coiled-coil signal of HR1 predicted by bioinformatics tools (see Figure S1) started to drop. On the other hand, we observed that HR1

helical content was increasing in presence of liposomes (Fig 6), indicating direct HR1/lipid interactions and aH folding. Moreover, docking and fusion experiments (including new data presented in the revised version of our manuscript) demonstrated the importance of this aH for membrane binding and fusion.
To search for a putative aH in HR2, we followed the same strategy, *i.e.* scanning the HR2 sequence with Heliquest (using a sliding window of 18 residues). We found many possible aH with very high hydrophobic moment all along the HR2 sequence. This result is expected because HR2 has a strong coiled-coil signature, which is by essence amphipathic thanks to the heptad repeats. However, in all the experiments we performed using HR2 and lipids, we did not detect any direct HR2/lipid interaction. Thus, we believe that there is no cognate helix within HR2. Its high amphipathicity is solely due to its coiled-coil signature.

2nd Editorial Decision 20 February 2018

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, all referees support publication in EMBO reports. Referee 1 however has some remaining concerns regarding the conclusion that HR2 dimerizes and drives membrane tethering in trans. Please either provide further biochemical evidence for HR2 dimers, as suggested by referee 1 or significantly tone down your conclusions on the tethering activity of HR2 and discuss potential limitations of the data also in light of recent publications, as indicated in your feedback. Please adjust the model shown in Figure 8 to discuss and show alternative models of membrane fusion. You might also want to focus the title more on HR1 and soften the conclusions on HR2 in the abstract. Please also provide the SNARE controls in Figure 4, as suggested by this referee and include the relevant controls when testing membrane insertion of the Mfn mutants.

Browsing through the manuscript myself, I noticed a few editorial things that we need before we can proceed with the acceptance of your study.

- Please reformat the references to fit the style of EMBO reports ('et al' should be used if there are more than ten authors, i.e., the first ten authors should be listed). You can download the respective EndNote file from our Guide to Authors

- The scale bars are very thin (e.g. in Fig 1C) and might be difficult to see in the final version. Also the figure labels of e.g. Figure 1D are rather small. Please ensure that all symbols and labels have a height of 1.5 to 2.0 mm at their final published size (font size: circa 7-8 pt at final size). See also our Figure guidelines

- Please add page numbers to the Appendix table of content and provide the Appendix as pdf file.

- Our data editors from Wiley have already inspected the figure legends for completeness. Please find their suggested changes in the attached Word file.

- Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large. For the larger image the height is variable. You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

We look forward to seeing a final version of your manuscript as soon as possible. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

The authors have responded to many of my concerns, and I continue to assert that studies like these are extremely important to provide mechanistic advances in the field. The functional interactions of HR1 with (even protein-free) membrane bilayers that drive both tethering and fusion is very convincing and important, and I appreciate their work for the revision providing titrations, additional controls, etc. However I would insist on minimizing any primary conclusions that HR2 drives membrane tethering in trans, since this is based more on assumptions from previous work rather than directly from evidence presented here. The recently solved crystal structures did not see HR2 interactions in trans, and the Cao paper very clearly showed trans interactions were achieved through the GTPase dimerization between opposing molecules (Cao et al., Nature 2017 PMID:28114303). In addition, a new study claims that HR2 resides within the intermembrane space, further questioning previous assumptions (Mattie et al., JCB 2017 PMID:29212658). Certainly HR2 may still form interactions in the IMS, or perhaps even downstream of conformational changes following GTPase mediated tethering, as suggested by the authors in their "alternative" supplementary model. But why favor such a direct model of the antiparallel coiled coil HR2 interaction between liposomes (that was effectively published in 2004)? There is no evidence anywhere in the manuscript that HR2 forms a dimer, even when tested directly by CD, non-denaturing gels, etc. Overall, I think that the major conclusion asserted by the authors that HR2 plays a central role in mitochondrial tethering must be supported by strong, direct evidence, or they should update their models and focus on the important findings related to HR1. Indeed, since HR1 is so sufficient in driving liposome tethering AND fusion, why the need for HR2 to tether as well?

My suggestions are below:
- The authors must biochemically confirm that HR2 is forming dimers that drive tethering, as predicted in Figure 4 (and the model in Figure 8). In the rebuttal the authors admit that others cannot see any HR2:HR2 interactions from the C-term domain, so why does the anchoring of these domains to liposomes trigger strong, functional trans dimers? The initial Chan paper from 2004 was a highly truncated version of HR2 containing a number of point mutations that allowed them to get a structure, and they did not show directly that this was responsible for tethering membranes together. In addition, this interaction was not observed in either of the new crystal structures by the Hu PMID:27920125 or Gao PMID:28114303, and, as stated above, the topology analysis from the McBride group directly contradict this model PMID:29212658. The authors use CD to monitor changes in HR1 with liposomes, and for reviewers in R5, but surely this can be used to confirm dimerization status of HR2, in combination with co-IP or other methods.

- Fig 4B, right graph shows a minor shift in diameter, from 35% with 30nm diameter to 28% with ~40nm diameter. The EM shows some liposomes in pairs, but without any clustering. In addition, there are doublet liposomes seen in the negative control, prompting some concerns over potential variation in sample preparation. The SNARE controls should be included throughout Fig 4, and the protein complexes isolated to capture the complexes in trans. To my eye, it is difficult to extrapolate these data into a solid conclusion that HR2 is a tethering peptide.

- As a minor, but important point, the confirmation of membrane insertion of the Mfn mutants did not include any internal controls of mitochondrial proteins peripherally associated with membrane, soluble in the matrix, and other transmembrane proteins.

Referee #3:

The authors carefully and fully responded to my comments.

2nd Revision - authors' response 7 March 2018

Specific answers to key reviewer 1's comments

Q1: However I would insist on minimizing any primary conclusions that HR2 drives membrane tethering in trans, since this is based more on assumptions from previous work rather than directly from evidence presented here.

A1: We have followed this advice and significantly modified our manuscript to focus our findings on the mode of action of HR1 in mitochondrial fusion. We however want to stress that our study shows using 3 independent approaches (magnetic bead docking assay, dynamic light scattering and electron microscopy) that isolated HR2 fragments mediate liposome docking *in vitro*. Liposome docking is not observed when either or both membranes do not contain any HR2 domain. Since HR2 does not interact with the membrane (as shown by liposome co-floatation and circular dichroism experiments), we conclude that – in our experiments with liposomes – docking originates from the formation of *trans*-HR2 complexes (see also our specific answer A4 below).

Q2: In addition, a new study claims that HR2 resides within the intermembrane space, further questioning previous assumptions (Mattie et al, JCB 2017, PMID:29212658).

A2: This recent study provides in fact compelling evidence that HR2 can be found in the mitochondrial intermembrane space, but it also conflicts with the work by several other labs, including the recent crystal structure of Mitofusin. The findings supporting a cytosolic orientation of the C-terminal HR2 are: (1) interactions between the HR1 and HR2 domains of Mitofusin, which cannot occur across the outer membrane (Rojo et al, J Cell Sci 2002, PMID:11950885; Honda et al, J Cell Sci 2005, PMID:15985463; Huang et al, PlosOne 2011, PMID:21647385); (2) protease sensitivity of N- and C-terminal domains of Mitofusin (Rojo et al, J Cell Sci 2002, PMID:11950885); (3) assembly of the HR2 domain within the N-terminal four-helix bundle that is adjacent to the cytosolic GTP-binding domain of the crystallized minimal Mitofusin GTPase domain and is required for the functional integrity of this fragment (Qi et al, J Cell Biol 2016, PMID:27920125; Cao et al, Nature 2018, PMID:28114303); and (4) the ability of ER-targeted Mitofusin to functionally replace Atlastin, an ER fusion protein exposing both N- and C-terminal domains towards the cytosol (Huang et al, PNAS 2017, PMID:29093165). We feel that the conclusions of these reports do not represent "assumptions" and that the data are at least as strong as those proposing an alternative topology.

Q3: But why favor such a direct model of the antiparallel coiled coil HR2 interaction between liposomes (that was effectively published in 2004)?

A3: We agree that there is no reason to favor one model over the other, notably because the studies of (Koshiba et al, Science 2004, PMID:15297672) and (Cao et al, Nature 2018, PMID:28114303) – which support HR2- and GTPase-mediated docking, respectively – both employed the same experimental strategy (point mutations to destabilize the dimers interface) to show that these dimers are important for mitochondrial docking *in situ*. We have thus significantly modified several parts of our manuscript (title, abstract, last section of the discussion, and final Fig. 8) in order to better focus our findings on the function of HR1 in mitochondrial fusion.

Q4: Fig 4B, right graph shows a minor shift in diameter, from 35% with 30nm diameter to 28% with ~40nm diameter. The EM shows some liposomes in pairs, but without any clustering. In addition, there are doublet liposomes seen in the negative control, prompting some concerns over potential variation in sample preparation. The SNARE controls should be included throughout Fig 4, and the protein complexes isolated to capture the complexes in trans. To my eye, it is difficult to extrapolate these data into a solid conclusion that HR2 is a tethering peptide.

A4: The shift in diameter may appear small but it actually corresponds to the expected $(2)^{1/3}$ fold increase resulting from the fusion of two liposomes with the same initial size. Similar size shift was observed for example by EM during the fusion between v-SNARE and t-SNARE liposomes or between synaptic vesicles and t-SNARE liposomes (Schuette et al, PNAS 2004, PMID:14981239; Holt et al, Curr Biol 2008, PMID:18485705). For the negative control, we chose a picture that was representative of our statistical analysis with three independent liposome preparations. It is common to observe some residual docking between protein-free liposomes, especially when these liposomes do not bear any charge, as is the case here. Note also that the presence of proteins should reduce docking if these proteins do not interact with each other (because they would instead add some steric repulsion between membranes). Instead, the amount of docked liposomes increase in the presence of HR1 or HR2, and the results are statistically different from those obtained in the absence of any HR domain. We did not include the SNARE controls in Fig. 4B because it has already been shown by us and others (using EM) that SNARE proteins mediate liposome docking to a similar extent (20-30% in Shen et al, Cell 2007, PMID:17218264; 40-50% in Hernandez et al, Science 2012, PMID:22653732; 30-40% in Park et al, J Biol Chem 2014, PMID:24778182). All samples in Fig. 4B were prepared using identical conditions chosen to obtain images that are free of artefacts (*e.g.* uranyl acetate concentration reduced to 1% and liposomes diluted down to 25 μM before observation). Because observations of liposomes by negative staining EM must always be interpreted with caution (Rigaud and Levy, Methods Enzymol 2003, PMID:14610807; Baxa, Methods Mol Biol 2018, PMID: 29039095), our results have been crosschecked with two other methods (dynamic light scattering and magnetic bead pull-down assays). We have modified the section of our manuscript presenting the EM data to include the different points mentioned above.

EMBO PRESS

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

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Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2016-43637

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
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Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
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  - definition of 'center values' as median or average;
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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Results were considered significant when the obtained values differed more than 2-fold from the background/control conditions and/or when differences could be validated though statistical tests such as t-test.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
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3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Experiments presented in the main figures of the article were replicated by different experimentalists.
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Microscopy pictures were analyzed blindly (ignoring the sample identity)
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Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, data compared using t-test were independent, had symmetric distributions and displayed similar variances
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Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	References for antibodies generated and characterized by the authors were provided (page 29 of the main text and page 11 of the supplementary text).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Immortalized Mouse Embryonic Fibroblasts (MEFs) lacking the endogenous Mfn1 protein (Mfn1 KO MEFs) were a gift from David Chan (California Institute of Technology, Pasadena, USA). Specific absence of Mfn1 was confirmed by Western blot. Absence of mycoplasma was confirmed by fluorescence microscopy after DNA-staining with DAPI.

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